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(54) Title: ANTIPARASITIC AGENT

(57) Abstract: The invention provides methods for treating conditions associated with the presence of an intraerythrocytic parasitic protozoan in a host organism and medicaments for use in such methods comprising haptoglobin-related protein as the active agent.

ANTIPARASITIC AGENT

Plasmodium falciparum, the most lethal species causing human malaria, replicates in the human host by first parasitising liver cells and subsequently erythrocytes. The classic pathology associated with *falciparum* malaria, characterised by fevers, rigour and anaemia is a direct consequence of the invasion and growth of the parasite within human erythrocytes and subsequent expansion of the parasite population. The size of the infected erythrocyte population is a key factor in defining the severity of malarial disease and prognosis of infection.

Erythrocytes become infected when parasite stages known as merozoites attach to and invade these cells. Various phases of parasite development can be observed during the 48 hour erythrocytic cycle [Freeman, 1983], viz: Rings (1-16 hours); trophozoites (17-36 hours); schizonts (37-48 hours). Schizont rupture results in the release of daughter merozoites infectious for erythrocytes. The replication rate of each infected cell has been measured to be between 2 and 12 in a non-immune human host [Kwiatkowski, 1991]. These rates suggest that the efficiency of replication may be affected by the environment in the circulation. Mediators of the innate and acquired immune system, fever, competition for erythrocytes of the optimum maturity as well as physical parameters related to invasion of erythrocytes in the circulation, will all effect the efficiency of parasite replication.

It is possible to observe the erythrocytic cycle in an *in vitro* culture system developed by Trager and Jensen [Trager, 1976]. This culture system requires growth of the parasite in up to 10% human serum. During adaptation of new isolates of *P. falciparum* to *in vitro* culture it has been observed that not all

non-immune human sera (i.e. sera from individuals who have not been exposed to *P. falciparum*) allow adequate replication of the parasite (K.P. Day unpublished observations). A typical serum screen measuring the replication of the parasite over a period of 5 to 6 days is shown in Figure 1. Considerable variability in capacity to support the growth of newly adapted isolates of *P. falciparum* is observed where replication rates range from 0.5 to 8. Occasionally lethal batches of sera have been identified. The detrimental effects of individual human sera can be observed by light microscopy to examine the health of trophozoite-infected erythrocytes at the 36 hour stage of development using the classification of Freeman [Freeman, 1983]. Good sera promote the growth of healthy trophozoites as revealed by the presence of both haemozoin pigment, indicating haemoglobin digestion, and a large food vacuole in the 36 hour infected cell. Parasites grown in toxic batches of sera show low replication rates and trophozoite formation does not proceed normally. Minimal or no haemoglobin digestion is observed in the 36 hour infected cell and no large food vacuole is observed.

Another parasitic protozoa, *Trypanasoma brucei brucei*, the causative agent of nagana in cattle has also been shown to be sensitive to human serum [Rifkin, 1978]. This species will not infect humans. In contrast, *T. brucei rhodesiense* and *T. brucei gambiense*, the causative agents of African sleeping sickness in humans, are resistant to the lytic effects of human serum. This host specificity of African trypanosomes appears to be defined by human serum sensitivity to a subclass of human high density lipoproteins (HDLs) called trypanosome lytic factor (TLF) [Hajduk, 1989]. TLF is believed to kill blood stream forms of *T. brucei brucei* by oxidative damage

initiated by its peroxidase activity [Hajduk, 1994]. TLF has recently been biochemically characterised and the major toxic component for trypanosomes has been shown to be the human haptoglobin-related protein (Hpr) [Smith, 1995]. The lytic effect of Hpr can be inhibited by the serum protein haptoglobin and thus the ratio of Hpr to haptoglobin may define the lytic activity of any human serum for *T. brucei brucei* [Smith, 1995]. More recently Hpr has been found to associate with serum proteins, including IgM, to form a high molecular weight complex designated TLF2 [Raper, 1996]. The relative importance *in vivo* of these two Hpr-containing complexes, i.e. TLF1 (HDL-associated TLF) and TLF2, remains to be established [Tomlinson, 1996].

The present inventors have now observed that the apolipoprotein Hpr in human HDL also has a direct toxic effect on the development of the erythrocytic stages of *P. falciparum*. These findings present a novel method of killing *P. falciparum* at the asexual intraerythrocytic stages and thus provide the basis for the development of a novel class of antiparasitic drugs active against non-trypanosomal parasitic protozoa.

Therefore, in accordance with a first aspect of the invention there is provided a method of treating or preventing a condition associated with the presence of an intraerythrocytic parasitic protozoan in a host organism, which method comprises administering to a host organism in need thereof an effective amount of a medicament comprising an active substance comprising a mammalian haptoglobin-related protein or a functional equivalent, functional fragment or structural analog thereof and a pharmaceutically acceptable carrier, diluent or excipient therefor.

The invention also provides for use of an active substance comprising a mammalian haptoglobin-related

protein or a functional equivalent, functional fragment or structural analog thereof for the manufacture of a medicament for use as an antiparasitic agent against intraerythrocytic parasitic protozoa or for use in the treatment or prevention of a condition associated with the presence of an intraerythrocytic parasitic protozoan in a host organism.

The invention further provides a method of treating or preventing a condition associated with the presence of an intraerythrocytic parasitic protozoan in a host organism, which method comprises administering to a host organism in need thereof an effective amount of a medicament comprising an active substance comprising at least one of:
(i) a mammalian HDL containing haptoglobin-related protein,
(ii) a mammalian TLF1, or
(iii) a mammalian TLF2,
or a combination thereof, optionally in combination with a mammalian haptoglobin-related protein or a functional equivalent, functional fragment or structural analog thereof and a pharmaceutically acceptable carrier, diluent or excipient therefor.

The invention still further relates to use of an active substance comprising at least one of:
(i) a mammalian HDL containing haptoglobin-related protein,
(ii) a mammalian TLF1, or
(iii) a mammalian TLF2,
or a combination thereof, optionally in combination with a mammalian haptoglobin-related protein or a functional equivalent, functional fragment or structural analog thereof, for the manufacture of a medicament for use as an antiparasitic agent against intraerythrocytic parasitic protozoa or for use in the treatment or prevention of a condition associated with

the presence of an intraerythrocytic parasitic protozoan in a host organism.

The experimental examples included herein demonstrate the antiparasitic activity of Hpr-containing HDL in an *in vitro* culture system for the malaria parasite *Plasmodium falciparum*. *P. falciparum* is known to require HDL for growth *in vitro* and appears to take up this lipid fraction of human serum in a stage-specific manner [Grellier, 1991]. On the basis of the data presented herein it is to be expected that Hpr is also capable of killing other intraerythrocytic parasitic protozoa, particularly those which actively take up HDL.

The methods and medicaments of the invention are particularly suitable for the treatment of conditions associated with intraerythrocytic parasitic protozoa from the phylum Apicomplexa, more specifically parasites belonging to the genus *Plasmodium*, including *Plasmodium faciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale, Plasmodium* species of rodents, *Plasmodium* species which parasitise birds and *Plasmodium* species which parasitise primates and also parasites belonging to the genus *Babesia*.

As used herein, the term "intraerythrocytic parasite" describes a parasitic organism which parasitises the erythrocytes of its host organism at some stage of its life cycle. An important example is the malaria parasite *Plasmodium falciparum* which replicates in a human host by first parasitising liver cells and subsequently erythrocytes, also having extracellular stages known as sporozoites and gametes. In contrast, the African trypanosome *T. brucei brucei* is exclusively extracellular and found mainly in the bloodstream and lymphatic system of its mammalian host. For the avoidance of doubt, the terms

"host" or "host organism" as used herein are to be interpreted as having their normal meaning within the field of parasitology.

The finding that a substance which is capable of killing *T. brucei brucei*, i.e. Hpr-containing HDL, is also active against intraerythrocytic parasites is unexpected because in order to mediate killing of an intraerythrocytic parasite Hpr-containing HDL must be trafficked through four membrane systems, the erythrocyte membrane and an outer membrane of the parasite (most probably the food vacuole membrane in *P. falciparum*). In contrast, Hpr-containing HDL taken up by the extracellular *T. brucei brucei* may be concentrated directly to the lysosome (see below). It is suspected that there may be a functional difference between Hpr-containing HDL uptake and/or killing mechanisms of the exclusively extracellular *T. brucei brucei* and intraerythrocytic parasites such as *P. falciparum*.

The medicaments and methods of the invention are useful not only in the treatment of conditions associated with the presence of an intraerythrocytic parasite in a human host but also with the treatment of conditions associated with the presence of an intraerythrocytic parasite in non-human host organisms, especially mammals and birds, in the field of veterinary medicine. For example, the method may be used in the treatment of infection with *Babesia* spp, particularly babesia infections of cattle, sheep, horses, goats, dogs and deer, and also in the treatment of *plasmodium* spp infections in birds.

In an extremely important aspect, the method of the invention can be used in the treatment of malaria associated with the presence of parasites of the genus *Plasmodium*, especially *Plasmodium falciparum*. The method of the invention offers considerable advantages

over conventional drug-based malaria treatments, not least because *in vitro* data suggest that it may provide an acute treatment that can be administered intravenously which works within a single erythrocytic cycle of infection. Where applicable, the method of the invention can be also be used in conjunction with any other known malaria treatment. In addition, the method of the invention may be used for prophylactic treatment to prevent or delay the onset of parasitic infection and/or the development of malarial symptoms. For the avoidance of doubt, it is to be understood that the term "malaria" as used herein encompasses both infection, particularly infection in the absence of disease symptoms, and malarial disease.

In addition to the treatment of conditions associated with infection with intra-erythrocytic parasitic protozoa, the medicament and method of the invention may also be used in the treatment of infection with non-protozoan single cell pathogens, including bacteria, viruses and fungi. Accordingly, the term "parasite", which is generally understood to refer to an organism which lives on or in the body of another (the host), is to be given a broad interpretation to include bacteria, viruses and fungi as well as intraerythrocytic protozoans.

"Treating" or "treatment" as used herein with reference to a given medical condition describes the management or care of a patient for the purposes of combatting the disease, condition or disorder and includes the administration of a medicament according to the invention to prevent the onset of the symptoms or complications (including prophylactic treatment), alleviating the symptoms or complications, or eliminating the disease, condition or disorder.

The phrase "effective amount" is taken to mean a therapeutically effective amount. The exact dosage and frequency of administration of a therapeutically

effective amount of a medicament according to the invention may depend on such factors as the form of the active substance, the dosage form in which it is administered and route of administration, the particular condition to be treated, the severity of the condition being treated and the age, weight and general physical condition of the patient, as would be appreciated by those skilled in the art.

According to the present invention, a medicament with antiparasitic activity for use in the treatment or prevention of a condition associated with the presence of an intraerythrocytic parasite in a host organism may be prepared from an active substance comprising a mammalian haptoglobin-related protein or a functional equivalent, functional fragment or structural analog thereof. Such a medicament may also be prepared from an active substance comprising at least one of:

(i) a mammalian HDL containing haptoglobin-related protein,

(ii) a mammalian TLF1, or

(iii) a mammalian TLF2,

or a combination thereof, optionally in combination with a mammalian haptoglobin-related protein or a functional equivalent, functional fragment or structural analog thereof. The term "active substance" as used herein may be taken as referring to a pharmaceutically active substance or agent. The term "pharmaceutical" should be taken to encompass compositions suitable for and intended for use in veterinary medicine in addition to human medicine.

The mammalian haptoglobin-related protein (Hpr) to be included in the medicament is preferably of primate origin and is most preferably of human origin. Human Hpr has been described previously in the literature, see for example Maeda, N. (1985) Nucleotide sequence of the haptoglobin and

haptoglobin-related gene pair. The haptoglobin-related gene contains a retrovirus-like element. *J Biol. Chem.*, 260, 6698-6709; Smith, A.B. *et al.* (1995) Killing of trypanosomes by the human haptoglobin-related protein. *Science*, 268, pp.284-286; Muranjan, M. *et al.* (1998) Characterization of the human serum trypanosome toxin, haptoglobin-related protein. *J Biol. Chem*, 273, pp.3884-7. The protein is encoded by the HPR gene located in the 16q22.1 region of human chromosome 16 (see Erickson, L. M. *et al.* (1992) Junctions between genes in the haptoglobin cluster of primates. *Genomics*, 14, pp 948-958).

Hpr protein isolated from normal human serum exists predominantly as a single α-β dimer derived from a precursor protein comprising the sequence of amino acids shown in Figure 4. This precursor protein is cleaved at the position indicated in Figure 4 to generate the separate α and β chains. The complete nucleotide sequence of the coding region of the human HPR gene is publicly available, see Genbank database accession numbers M69197 and K03431.

An alternative, cancer-related form of a human Hpr-like protein has also been reported (Tabak, S. *et al.* (1996) Transcriptionally active haptoglobin-related (Hpr) gene in hepatoma G2 and leukemia molt-4 cells. *DNA Cell Biol*, 15, pp.1001-7). This form of the protein contains an N-terminal extension not found in Hpr present in normal human serum (see full protein sequence deposited under Genbank accession number X89214). The complete genomic sequence of the HPR locus on human chromosome 16, including the intergenic region between the 3' end of the HP gene and the 5' end of the HPR gene, is deposited under Genbank accession number M69197.

It is also within the scope of the invention to use a non-human mammalian homologue of Hpr as the

active substance in the preparation of a medicament according to the invention. Hpr proteins falling within the definition of a "mammalian Hpr protein" will typically share at least 30% amino acid sequence identity with the human Hpr protein, and more preferably will be at least 40%, 50%, 60%, 70%, 80%, 90% or 95% identical to the human Hpr protein. Amino acid sequence identity (%) is calculated on the basis of an optimal alignment of the sequences to be compared, taking into account insertions or deletions. Optimal sequence alignments may be assembled using one of the computer algorithms known in the art, such as BLAST (accessible via www.ncbi.nlm.nih.gov) or FASTA.

Advantageously, the non-human Hpr homologue may be of primate origin. The existence of Hpr homologues in non-human primates, specifically apes and Old World monkeys is well documented (see McEvoy, S.M. and Maeda, N. (1988) Complex events in the evolution of the haptoglobin gene cluster in primates. *J Biol Chem*, 263, pp 15740-7). Moreover, sera from non-human primates that contain an HPR gene, with the exception of Chimpanzee serum, have been shown to be trypanolytic (Seed, J.R. *et al.* (1990) *J. Protozool*, 37, 393-400; Hawking, F. (1973) *Trans. R. Soc. Trop. Med. Hyg.* 67, 501-517). It is therefore to be expected that Hpr proteins isolated from non-human primates, particularly species whose sera are known to be trypanolytic such as gorilla, mandrill and baboon, will also exhibit antiparasitic activity against intraerythrocytic protozoan parasites such as *P. falciparum*.

Knowledge of the primary nucleotide sequence of the human HPR gene and cDNA facilitates the cloning of homologous HPR genomic and/or cDNA sequences from non-human primates, and indeed from other non-primate mammalian species in which HPR homologues are present.

For example, a labelled probe based on a fragment of the human Hpr cDNA could be used to screen a genomic or subgenomic library from the species in question, or a cDNA library prepared from mRNA isolated from a tissue or cell type known to or thought to express Hpr, in order to identify positive clones containing Hpr sequences. Procedures for the preparation of suitable probe fragments, construction of genomic, subgenomic and cDNA libraries, cross-species library screening, recovery of positive clones and sequencing of the DNA inserts would be well known to one skilled in the art (see, for example, Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; F. M. Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994)). As an alternative to the library screening approach, oligonucleotide primers corresponding to suitable regions of the human Hpr cDNA sequence could be used for PCR amplification of homologous HPR sequences from non-human genomic DNA or RT-PCR amplification from non-human total or poly A+ RNA, this RNA preferably being isolated from a tissue known or thought to express Hpr.

Skilled artisans will recognise that mammalian Hpr proteins for use in the manufacture of a medicament according to the invention can be synthesised by a number of different methods, such as chemical methods known in the art or recombinant methods.

Hpr proteins for use in the invention are preferably produced by recombinant DNA methods using cloned DNA fragments encoding the Hpr protein (e.g. a cloned cDNA). As would be well known to those of ordinary skill in the art, the basic steps in the production of a recombinant protein are: provision of a DNA molecule encoding the Hpr protein, integrating

the DNA into a replicable expression vector in a manner suitable for expressing the Hpr protein either alone or as a fusion protein, introducing the expression vector into a suitable host cell, culturing the host cell under conditions which promote expression of the Hpr protein and recovering and substantially purifying the Hpr protein.

Techniques for solid phase chemical synthesis of proteins are well known in the art and could be used as an alternative to the recombinant expression approach (see Merrifield, R.B. (1963), Automated Synthesis of Peptides, Science 150: pp178-184). By way of example, polypeptides may be synthesised on an Applied Biosystems 430A peptide synthesiser using reagents and protocols supplied by the manufacturer (Applied Biosystems).

It is also within the scope of the present invention to use as the active substance in the manufacture of a medicament according to the invention a functional equivalent, functional fragment or structural analog of a mammalian haptoglobin-related protein.

A "functional fragment" of an Hpr protein is conveniently identified as a fragment of an intact Hpr protein that retains antiparasitic activity over the required host range either in an *in vitro* test system or *in vivo*, for example in an animal model of the parasitic infection. Functional fragments may be produced by a synthetic method, by chemical or enzymatic cleavage of an intact Hpr protein or, most preferably, by recombinant DNA techniques.

"Functional equivalents" of a given Hpr protein include variant forms having substantially the same biological function as the 'wild type' Hpr protein. In the context of this application, the "biological function" of an Hpr protein is defined to mean the ability to kill intraerythrocytic parasitic protozoa

of the phylogenetic origin identified herein in an *in vitro* or *in vivo* test system, for example the *P. falciparum* test system described herein. An Hpr "functional equivalent" has substantially the same biological function as the wild-type Hpr protein if it retains the ability to mediate killing of intraerythrocytic parasites, although its killing activity may be less than or greater than the killing activity of the wild-type Hpr protein. Changes which are conservative of Hpr function may include insertion or deletion of one or more amino acids or conservative substitution of one or more amino acids with another amino acid or acids having similar chemical characteristics, substitution with an unusual amino acid residue and also *in vivo* or *in vitro* chemical and biochemical modifications, such as acetylation, carboxylation, phosphorylation and glycosylation. The choice of amino acids for making conservative changes will be well-known to those skilled in the art.

"Functional equivalents" of a given Hpr protein may also include fusion proteins, for example fusions comprising the full length Hpr protein or a functional fragment thereof fused either N-terminally or C-terminally to an heterologous protein or peptide fragment or fusions comprising Hpr or a functional fragment thereof having heterologous proteins or peptide fragments fused to both the - and C-termini.

Fusion proteins will typically be made by recombinant nucleic acid techniques in which two or more open reading frames are translationally fused or may be chemically synthesized. In recombinant systems, expression of a fusion protein can provide a convenient means for purification, the heterologous protein or peptide tag commonly being removed after purification by chemical or enzymatic cleavage. An Hpr protein or a functional fragment thereof could also be fused to a heterologous protein or peptide

which imparts an additional function, such as for example binding to a receptor found on the surface of the parasite or on the surface of the parasitised cell. This could provide a convenient means for facilitating entry of a functional Hpr protein into the parasite, for example via a receptor-mediated endocytic pathway.

"Functional equivalents" of a given Hpr protein also include structural analogs thereof with equivalent biological function. Biologically active Hpr analogs can be designed and produced according to techniques known to those of skill in the art (see. e.g., US Patent Nos 4,612,132; 5,643,873 and 5,654,276). These structural analogs can be based, for example, on a specific Hpr amino acid sequence and maintain the relative position in space of the corresponding amino acid side chains. In a preferred embodiment, the structural analog retains the biological function of the wild-type Hpr, as defined herein, but possesses a "biological advantage" over the corresponding wild-type Hpr amino acid sequence with respect to one or more of the following properties: solubility, stability and susceptibility to hydrolysis and proteolysis.

Methods for preparing Hpr structural analogs include modifying the N-terminal amino group, the C-terminal carboxyl group, and/or changing one or more of the amino linkages to a non-amino linkage. Two or more such modifications can be present in a single structural analog molecule. Modifications of peptides to produce structurally analogous molecules of equivalent biological function are described in US Patent Nos. 5,643,873 and 5,654,276.

References hereinbelow to "Hpr protein(s)" in connection with the preparation of pharmaceutical formulations in accordance with the invention should, unless otherwise stated, be taken to include all

possible functional fragments, functional equivalents and structural analogs, as defined herein.

In addition to isolated mammalian Hpr proteins, HDL itself, TLF1 or TLF2 may be used as the active substance in a medicament according to the invention.

In general, mammalian serum lipoproteins consist of a core of neutral lipids and a surrounding shell of phospholipids with embedded apolipoproteins. Each class of lipoprotein has a characteristic density that depends on the ratio of protein to lipid, and each has a characteristic complement of lipids and apolipoproteins. Human HDL particles generally have a density of about 1.063-1.210 g/ml and a diameter of about 9-12nm and contain the major apolipoproteins ApoAI and ApoAII. An HDL fraction containing haptoglobin-related protein and thus having anti-parasitic activity against intraerythrocytic parasitic protozoa can be prepared from normal human serum by density gradient centrifugation according to standard procedures known in the art (Hajduk, S.L. *et al.* Lysis of *Trypanosoma brucei brucei* by a toxic subspecies of human high density lipoprotein. *J. Biol. Chem.* **264**, 5210-5217 (1989)). The antiparasitic activity of the HDL fraction thus obtained can be tested either *in vitro* (e.g. using the *in vitro P. falciparum* culture system described in the examples given herein) or *in vivo* , for example in an animal model.

TLF1 (also known as HDL-associated TLF) may be purified from normal human serum using the procedure of Hajduk, S.L. *et al.* (1989) Lysis of *Trypanosoma brucei* by a toxic subspecies of human high density lipoprotein. *J. Biol. Chem.* 264, 5210-17. TLF1 purified according to this procedure comprises unusually large HDL particles with a diameter of 18-22 nm, consistent with an average particle size of approximately 500 kDa, and has a particle density of

about 1.21-1.24 g/ml. TLF1 may also be purified from human serum using a density gradient purification procedure described by Muranjan, M. *et al.*, *J Biol. Chem.*, 273, 3884-3887. Alternatively, both TLF1 and TLF2 (non-HDL associated TLF) can be purified by an immunoaffinity-based purification procedure described by Raper, J. *et al.*, Characterization of a novel trypanosome lytic factor from human serum. *Infec. Immun.*, 67, 1910-6. Again, the antiparasitic activity of a purified TLF preparation can be tested either *in vitro* (e.g. using the *in vitro P. falciparum* culture system described in the examples given herein) or *in vivo*, for example in an animal model of parasitic infection.

As an alternative to HDL, TLF1 or TLF2 prepared from serum, it is also within the scope of the invention to use reconstituted HDL or TLF particles which display similar structure and antiparasitic activity to the naturally occurring particles. Methods for reconstituting lytic HDL particles are described by Tytler, E.M., *et al.* (1995) Reconstitution of the trypanolytic factor from components of a subspecies of human high-density lipoproteins. *Mol Biochem. Parasitol.*, 69, pp. 9-17.

The natural "packaging" of the toxic agent Hpr within HDL or TLF1 particles provides a convenient route for delivery of Hpr, for example to an intracellular parasite, via natural pathways for HDL/TLF1 uptake. Accordingly, in one embodiment of the invention an antiparasitic medicament can be formulated from HDL particles or TLF1 particles or a combination thereof together with a pharmaceutically acceptable diluent, carrier or excipient therefor. In this context, the terms "HDL particles" and "TLF1 particles" encompass not only naturally occurring forms purified from serum but also reconstituted Hpr-

containing HDL or TLF particles assembled *in vitro*. Convenient dosage forms may include an injectable/infusable formulation for direct intravenous administration. For therapeutic purposes, the formulation could be administered in a single daily dose or in multiple doses per day. The amount per administration will be determined by a physician and depend on such factors as the nature and severity of the disease, and the age and general condition of the patient.

Hpr can also be administered using "non-natural" delivery systems and it is to be understood that the invention is in no way limited to the administration of Hpr in the form of naturally occurring HDL or TLF1.

In one embodiment, an Hpr protein can be combined with pharmaceutically acceptable carriers, diluents or excipients. For intravenous use, the Hpr protein may be administered in commonly used intravenous fluid(s), for example physiological saline, Ringer's solution or 5% dextrose, and administered by direct injection or infusion. Other routes of administration of an Hpr protein based drug are also contemplated, e.g. transdermal administration, inhalation or even oral delivery.

Conveniently, the Hpr protein may also be incorporated into lipid micelles or liposomes which are then formulated with a pharmaceutically acceptable diluent or carrier. Further protein components in addition to Hpr may be incorporated into the micelles or liposomes.

In a further aspect, the invention provides a method of treating or preventing a condition associated with the presence of an intraerythrocytic parasite in a host organism, which method comprises administering to a host organism in need thereof an effective amount of a medicament comprising an expression vector suitable for directing expression of

a mammalian Hpr protein or a functional fragment or functional equivalent thereof in the host organism.

Consistent with this aspect of the invention, a nucleic acid molecule encoding a mammalian haptoglobin-related protein or a functional fragment or functional equivalent thereof may be used for the manufacture of a medicament for use as an antiparasitic agent against intraerythrocytic parasitic protozoa or for use in the treatment or prevention of a condition associated with the presence of an intraerythrocytic parasitic protozoan in a host organism.

Preferred types of expression vectors for *in vivo* use are viral vectors, particularly adenovirus-derived vectors or retrovirus-derived vectors, although plasmid expression vectors have also been proposed for use in somatic gene therapy. A number of suitable viral and plasmid vectors are known in the art which are suitable for use in mammalian hosts.

In a still further aspect, the invention provides a method of identifying a compound with potential antiparasitic activity against intraerythrocytic parasitic protozoa, which method comprises the steps of:

(a) providing a eukaryotic host cell containing a reporter gene expression construct comprising the promoter region of the human HPR gene operably linked to a reporter gene;

(b) contacting said recombinant host cell with a test compound; and

(c) screening for expression of the reporter gene product.

wherein compounds which increase expression of the reporter gene product are scored as having potential antiparasitic activity.

The above method of the invention can be used to identify compounds which up-regulate the expression of Hpr and hence have potential antiparasitic activity against parasites which are sensitive to Hpr-mediated killing, for example the malaria parasite *Plasmodium falciparum*.

For the purposes of this application, the term "promoter region of the human HPR gene" may refer to the proximal promoter region, i.e. sequences immediately upstream of the Hpr transcription start site which are necessary for correctly positioning RNA polymerase and also to the proximal promoter region plus any additional sequence elements which may be involved in regulating Hpr gene expression, e.g. upstream enhancer sequences, intronic sequence elements etc.

It is to be expected that the HPR proximal promoter will be contained within the intergenic region which separates the HP and HPR genes on human chromosome 16. Accordingly, in one embodiment of the method of the invention the promoter region of the human HPR gene comprises the nucleotide sequence from position 7283 to position 9429 of the sequence illustrated in Figure 4 (Genbank accession number M69197) or a transcriptionally active fragment thereof.

The term "operably linked" as used herein refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. In this case, the promoter region of the human HPR gene, as defined above, is positioned to control expression of a reporter gene encoding a protein product which is directly or indirectly detectable. The juxtaposition of the HPR promoter region and a reporter gene may be referred to herein as a 'reporter gene expression construct'.

Reporter genes which may be used in accordance

with the invention include those which encode a fluorescent product, such as green fluorescent protein (GFP) or other autonomous fluorescent proteins of this type or those which encode an enzyme product, such as for example chloramphenicol acetyl transferase (CAT), β-galactosidase and alkaline phosphatase, which is capable of acting on a substrate to produce a detectable product.

Reporter gene assays using reporter gene expression constructs are well known in the art and commonly used in the art to test the promoter activity of a given DNA fragment. They may also be adapted, as in the present invention, to screen for compounds capable of modulating gene expression.

The reporter gene expression construct is preferably incorporated into a replicable expression vector so that it may be conveniently introduced into the eukaryotic host cell. The eukaryotic host cell must be one which contains the appropriate transcription machinery for RNA Polymerase II transcription, and is preferably a cultured mammalian cell. In a preferred embodiment, the host cell is a cell type which is known to express Hpr *in vivo* or is a transformed cell line derived from a cell type known to express Hpr *in vivo*.

An expression vector may be inserted into the host cell in a manner which allows for transient transfection or alternatively may be stably integrated into the genome of the cell (i.e. chromosomal integration). Chromosomal integration is generally preferred for drug screening because the expression constructs will be maintained in the cell and not lost during cell division, also there is no need to separately control for the effects of copy number.

Stable integration of a reporter gene expression construct into the genome of eukaryotic host cell may be achieved using a variety of known techniques. The

most simple approach is selection for stable integration following transfection of a host cell with a plasmid vector. Briefly, a plasmid vector comprising a reporter gene expression construct consisting of the HPR promoter region ligated to a promoterless reporter gene cDNA and also a gene encoding a dominant selectable marker, such as neomycin phosphotransferase, is first constructed using standard molecular biology techniques. The plasmid vector is then used to transfect eukaryotic host cells using one of the standard techniques such as, for example, lipofection. Following transfection stable cell lines in which the plasmid DNA has become randomly integrated into the chromosome are selected with growth on appropriate media. For plasmids carrying the neomycin phosphotransferase gene this is achieved using the antibiotic G418. Plasmid vectors suitable for use in the construction of stable cell lines are commercially available (for example the pCI-neo vector from Promega corporation, Madison WI, USA).

Stable integration into mammalian chromosomes may also be achieved by homologous recombination, a technique which has been commonly used to achieve stable integration of foreign DNA into embryonic stem cells as a first stage in the construction of transgenic mammals. Stable integration into eukaryotic chromosomes can also be achieved by infection of a host cell with a retroviral vector containing the appropriate reporter gene expression construct.

It will be appreciated that a wide variety of compounds can be tested using the method of the invention to see whether they are capable of up-regulating HPR gene expression and hence have potential antiparasitic activity. The compound may be of any chemical formula and may be one of known

biological or pharmacological activity, a known compound without such activity or a novel molecule such as might be present in a combinatorial library of compounds. The method of the invention may be easily adapted for screening in a medium-to-high throughput format.

Compounds which are identified as being capable of up-regulating HPR gene expression should be further tested in order to establish whether the effect on gene expression is HPR-specific or non-specific. This could be achieved using a control cell containing a control reporter gene expression construct with no HPR promoter sequences.

In a still further aspect, the invention provides for use of an antibody which binds preferentially to the human Hpr protein, as compared to the human haptoglobin protein, in a diagnostic test to determine the levels of Hpr protein in a clinical sample taken from a mammalian subject.

Antibodies which bind preferentially to the human Hpr protein, as compared to the human haptoglobin protein, are generally useful in diagnostic tests to determine levels of Hpr protein in samples of biological material (for example blood, serum, plasma, tissue biopsys etc) in a clinical, including veterinary medicine, context. In particular, the invention provides for use of an antibody which binds preferentially to the human Hpr protein, as compared to the human haptoglobin protein in a diagnostic test to determine whether an individual is likely to be susceptible or resistant to malaria. Similar diagnostic tests may be performed in order to determine susceptibility to other disease conditions for which serum levels of Hpr are a risk factor.

Experiments carried out by the present inventors indicate that serum levels of Hpr alone are important

in defining serum toxicity to intraerythrocytic *P. falciparum*. Therefore, it may be possible to use serum levels of Hpr as an indicator of susceptibility to malaria. In this context, the term "malaria" again encompasses both infection with *P. falciparum* and malarial disease. Individuals having high serum levels of Hpr protein (as compared to the average serum level of Hpr in an ethnically matched population) are more likely to be resistant to malaria, whilst those with low serum levels of Hpr protein (as compared to the average serum level of Hpr in an ethnically matched population) are more likely to be susceptible to malaria. This is in marked contrast to the situation with *T. brucei brucei* where the ratio of Hpr to haptoglobin may define the toxic activity of any human serum for this parasite, suggesting that there may be a functional difference between the TLF binding and Hpr uptake mechanisms of *T. brucei brucei* and *Plasmodium falciparum*.

In one embodiment the invention provides an *in vitro* method of determining whether an individual is likely to be susceptible or resistant to malaria, which method comprises contacting a sample of tissue or body fluid from said individual with an antibody which binds preferentially to the human Hpr protein as compared to the human haptoglobin protein and detecting or quantitatively measuring any complexes formed by binding of said antibody to haptoglobin-related protein present in the sample of tissue or biological fluid.

This method may be performed in any standard immunoassay format known in the art, such as an enzyme-linked immunosorbent assay (ELISA), a radioimmunoassay or the like. Suitable biological fluids to be tested may include whole blood, plasma, serum, urine, sweat, saliva etc. Advantageously, the

biological fluid on which the method is carried out may be pre-treated in order to reduce the amount of haptoglobin present in the sample. For example, the method may be carried out on haptoglobin-depleted serum.

A typically immunoassay would involve contacting a sample of biological fluid with the antibody to allow specific binding to any Hpr protein present in the sample and then detecting the presence of/measuring the amount of complexes of antibody bound to Hpr protein. As an alternative to an immunoassay, the method be performed in an immunohistochemistry format on a sample of tissue removed from the individual. Procedures for performing immunoassays or immunohistochemistry in accordance with this aspect of the invention would be well known to the skilled artisan and are described, for example, in IMMUNOASSAY: E. Diamandis and T. Christopoulus (1996), Academic Press, Inc., San Diego, CA; IMMUNOCHEMISTRY 1 and 2: A practical approach (1997), A.P. Johnstone and M.W. Turner, Eds., IRL Press at Oxford University Press.

Antibodies for use in the methods of the invention which preferentially bind to human Hpr, as compared to human haptoglobin, may be prepared using techniques which are known *per se* in the art, using a fragment of the Hpr protein as the challenging antigen. Advantageously, this fragment should correspond to a region of the Hpr protein which is divergent from human haptoglobin, i.e. an epitope which is not present on the haptoglobin molecule. Peptide fragments of the Hpr protein may synthesised by chemical synthesis techniques (see, for example, Merrifield, R.B. (1963), Automated Synthesis of Peptides, Science 150: pp178-184). In order to elicit a strong immune response the peptide fragment (also known as a hapten) may be covalently linked to a

carrier molecule such as bovine serum albumin, ovalbumin or Keyhole Limpet Hemocyanin (KLH), as is well known in the art. Anti-Hpr polyclonal antibodies may be prepared by inoculating a host animal, such as a rabbit, with an Hpr peptide-carrier conjugate and recovering immune serum and the same Hpr peptide-carrier conjugate could also be used as challenging antigen for the preparation of anti-Hpr monoclonal antibodies, according to standard techniques (see, for example ANTIBODIES: A Laboratory Manual, E. Harlow and D. Lane, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY; IMMUNOCHEMISTRY 1 and 2: A practical approach (1997), A.P. Johnstone and M.W. Turner, Eds., IRL Press at Oxford University Press).

The antibody used in the diagnostic methods of the invention may be any antibody, polyclonal or monoclonal, having a differential binding affinity for Hpr and for haptoglobin, the difference in binding affinity being sufficiently high enough for the antibody to distinguish between Hpr and haptoglobin under the conditions of the particular diagnostic method in question. The antibody will preferably have a binding affinity for the human Hpr protein at least ten times, more preferably at least 50 times, even more preferably at least 100 times greater than the binding affinity of the said antibody for the human haptoglobin protein. Most preferably the antibody will bind specifically to the human Hpr protein, with only background or non-specific binding affinity for human haptoglobin.

The present invention will be further understood with reference to the following non-limiting experimental example, together with the accompanying Figures in which:

Figure 1 illustrates serum and HDL toxicity for *P.*

falciparum.

Figure 1a: Ring stage parasites were cultured in RPMI medium containing 10% human serum, various different sera being denoted by the codes on the horizontal axis. Initial parasitaemia in each culture was 1%. The cultures were fed daily with the appropriate 10% sera in RPMI medium. After 5 days the parasitaemia in each culture was determined by flow cytometry. Parasitaemia varied depending upon the serum present in the medium.

Figure 1b: Late ring stage parasites at a parasitaemia of 1% were cultured with various concentrations of HDL (expressed as trypanosome lytic units/ml) for 24 hours. Parasites were then maintained until re-invasion had occurred at which time the % parasitaemia in each culture was determined by flow cytometry. The presence of HDL in the medium reduced parasitaemia in a dose-dependent fashion.

Figure 1c: Late ring stage parasites as a parasitaemia of 2% were cultured with HDL (H) (10,000 units/ml) or with phosphate buffer (controls (C)). Parasitaemia, parasite stage and health were then assessed by light microscopy at various time points from 12-30 hours after addition of HDL. Unhealthy parasites were shrunken, misshapen and/or had abnormal haemozoin pigment not associated with the food vacuole.

Figure 1d: Late ring stage parasites at a parasitaemia of 1% were cultured with ^{3}H-hypoxanthine and various concentrations of HDL (expressed as trypanosome lytic units/ml) for 24 hours. Cells were then harvested and incorporation of radioactive hypoxanthine into parasite nucleic acid determined by scintillation counting (expressed as counts per minute; CPM). The presence of HDL in the medium reduced nucleic acid synthesis in a dose-dependent fashion.

Figure 2 illustrates the effects of HDL determined by electron microscopy.

Figure 2a: Electron micrograph through an infected erythrocyte containing a mature schizont with developing merozoites (M) from the control sample at 30 hours post treatment. K-knob. Bar is 1Mm.

Figure 2b: Section through an infected erythrocyte from a sample 30 hours post treatment with HDL in which an intact early trophozoite is present which contains a few pigment crystals (P). Note the absence of knobs at the erythrocyte surface. Bar is 1µm.

Figure 2b insert: Detail of part of an infected erythrocyte from the HDL-treated sample showing a degenerate trophozoite which is recognisable by the presence of clumps of pigment crystals (P). Bar is 1µm.

Figure 2c: Graph showing the relative numbers of early, late and dead parasites in the control and treated groups at 20 hours post treatment as determined by electron microscopy.

Figure 3 illustrates the effects of HDL negative Hpr (HDL-Hpr).

Figure 3a: Characterisation of Hpr-depleted material by stained gel (top) and western blot (anti-Hp) (bottom) for purified Hp (lane 1), purified TLF (lane 2), proteins from total HDL bound to abti-Hp column and eluted (lane 3), proteins from total HDL following depletion on the anti-Hp column (lane 4) and the total proteins in the HDL preparation (lane 5). The position of the β-subunit of Hpr is indicated with an *. A protein of this size is enriched in lane 3 as expected. This band was purified and analysed and the mass and sequence of selected tryptic fragments by mass spectrometry characterised it as Hpr.

Figure 3b: Late ring stage parasites at a parasitaemia

of 1% were cultured with various concentrations of HDL (expressed as units/ml) with or without the Hpr-containing fraction for 24 hours. Parasites were then maintained until re-invasion had occurred at which time the % parasitaemia in each culture was determined by flow cytometry. The dose-dependent reduction in parasitaemia due to the presence of HDL did not occur following removal of Hpr from the HDL fraction.

Figure 4 illustrates the amino acid sequence of the human haptoglobin related protein (Hpr). The sequence shown is the amino acid sequence of a precursor polypeptide chain which is cleaved between residues R103 and I104 to generate separate α and β subunits.

Experimental methods

HDL purification and trypanosome lytic assay

The HDL fraction of human serum was prepared by ultracentrifugation and dialysis against phosphate buffered saline (PBS), according to the method of Hajduk, S.L. *et al* [7]. Freshly collected human blood was used to prepare 200 ml of serum. Intact serum was adjusted to 1.063 g/ml with sodium bromide and both low density lipoprotein and chylomicrons were removed by flotation. The pellet, rich in serum proteins and HDL, was adjusted to 1.26 g/ml with sodium bromide and total serum HDL further purified by centrifugation. The HDL was titrated in *P. falciparum* cultures according to the number of measured trypanosome lytic units. A unit is defined experimentally as the amount of HDL necessary to cause 50% lysis of 1x 10^7 *T. brucei brucei* in 2 hours at 37°C in a standard lysis assay [Hajduk, S.L. *et al. Journal of Biological Chemistry* **264**, 5210-5217 (1989)].

P. falciparum cultivation and growth inhibition assay

Isolates and cloned lines of *P. falciparum* were cultured according to the method of Trager and Jensen[5] as modified by Day et al [16] at 5% haematocrit in RPMI medium supplemented with 5 % human serum previously screened to support parasite growth. The parasites were synchronised by plasmagel selection [17] in one cycle and sorbitol treatment [18] in the next cycle. These sorbitol treated ring stage parasites were used to set up cultures at 1% parasitemia in 250 ml wells of 24 well plates (Sterilin, UK) unless otherwise stated. When cultures were at the mid to late ring stage (12 to 15 hours post invasion) HDL or equivalent volume of PBS was added to give a final concentration of 0 to 17,000 trypanolytic units/ml. The maximum test volume added was variable according to batches of HDL but never exceeded 30µl. The HDL was removed with the culture supernatant after 24 hours (late trophozoite stage) and replaced with complete medium [16] containing 5% serum. Parasite replication was assessed following parasite reinvasion, 60 hours after addition of HDL; infected erythrocytes were labelled with ethidium bromide, cultures were then fixed with 0.5% paraformaldehyde and parasitaemia determined using flow cytometry [19]. Nucleic acid synthesis during trophozoite maturation was assessed by measurement of ^{3}H-hypoxanthine uptake by parasite cultures using the LKB Walla cell harvester [20]. Visual determination of parasite health was made at various time points following addition of HDL using both light microscopy of Giemsa-stained cells and electron microscopy.

Light and electron microscopy of infected erythrocytes

Cultures of *P. falciparum* infected erythrocytes at the late ring stage (12-15 hours post invasion) were divided into aliquots and treated with various

concentrations of HDL or an equivalent volume of phosphate buffer (control) and cultured for a further 30 hours. Thin smears were prepared, fixed with methanol and stained with Giemsa for light microscopy. Other samples for electron microscopy were fixed in 2.5% gluteraldehyde in 0.1M cacodylate buffer. The samples were post-fixed in 1% osmium tetroxide, dehydrated in ethanol, treated with propylene oxide and embedded in Spurr's epoxy resin. Thin sections were stained with uranyl acetate and lead citrate prior to examination in the JEOL 1200EX transmission electron microscope. For quantitation, a random sample of 100 infected erythrocytes were examined and placed into one of three groups; early stages which comprised rings up to early trophozoites; late stages comprising mid-trophozites to mature schizonts and dead parasites exhibiting features consistent with irreversible degeneration. This was repeated for a minimum of four times for separate blocks from each sample.

Depletion of Hpr containing HDL by affinity chromatography

Anti-haptoglobin affinity resin was prepared by coupling 2.0 g of cyanogen bromide-activated Sepharose 4B (Pierce) with 20mg of rabbit anti-human haptoglobin (Sigma). Purified total human serum HDL was passed over the anti-haptoglobin column at 4°C, recycled for approximately 20 column volumes then washed with 50 volumes of PBSE. Hpr containing HDL was eluted with 4M guanidine-hydrochloride. The eluate was dialyzed overnight against EDTA-phosphate buffer. Samples of intact serum, 1.26 g/ml float, Hpr depleted HDL and HDL enriched in Hpr were separated on 12% SDS-PAGE and either Coomassie stained or transferred to nitrocellulose and probed with rabbit anti-human haptoglobin.

Sequence determination of Hpr

The purified HDL containing Hpr were separated on 12% SDS-PAGE, Coomassie stained and the 13.5kDa and 36kDa bands corresponding to the α and β subunits of Hpr were excised out of the gel. The proteins were eluted, digested with trypsin and portions of the unseparated digests analysed by MALDI (Perceptive Biosystem Voyager Elite Malditos Mass Spectrophotometer) [21].

Example 1

HDL mediated lysis of *P. falciparum*

Varying amounts of HDL were added to *P. falciparum* ring stage (12-15 hours post invasion) cultures. Figure 1b shows a dose response curve where HDL containing 6000 trypanosome lytic units reduced parasite invasion by 95% in the cycle after treatment. To determine which life cycle stages were sensitive to the toxic effect of the HDL, this fraction of human serum was added to cultures at different times. The presence of HDL in cultures between 16 to 40 hours post invasion (ie. during trophozoite maturation) prevented parasite replication as shown in Figure 1b. When HDL was added to late schizonts (44-48 hours post invasion) and present during schizont rupture and merozoite release there was no reduction in re-invasion and growth in the following cycle indicating that schizonts and free merozoites were not sensitive to the toxic effects of HDL (data not shown). Data are presented in Figure 1b for passages of the recently-adapted isolate MUZ 37, although similar results were obtained for isolates 1776, Muz 106 and the long-term isolate 3D7 (data not shown). Light microscopic examination of Giemsa-stained smears was used to assess parasite growth and development

when the HDL fraction was present in cultures during trophozoite maturation (Figure 1c). No obvious changes in parasite development were observed 12 hours post HDL treatment. The detrimental effects of this treatment were observed after 18 hours (ie. in erythrocytes 30 to 33 hours post invasion). The parasitaemia was reduced by 50% compared to controls. The remaining trophozoites were small in size, had small food vacuoles and little evidence of pigment. The proportion of unhealthy trophozoites continued to increase 24 and 30 hours after addition of HDL. Susceptibility to the toxic effect of HDL was detectable during trophozoite maturation. This coincides with the time (21 to 36 hours post invasion) when maximum HDL uptake occurs as assessed by labelled PC uptake [11].

The toxic effect of HDL containing Hpr on parasite development was examined by electron microscopy 24 hours after addition to ring stage cultures (40 hours after erythrocyte invasion). In buffer-treated control samples, the majority (approximately 70%) of infected erythrocytes contain parasites at the mid trophozite to late schizont stage with a few mature schizonts (Figure 2a). Relatively few early stages (approximately 20%) and dead parasites (approximately 7%) were observed (Figure 2c). The parasites contained a large nucleus or multiple nuclei, rough endoplasmic reticulum, ribosomes, simple mitochondria and large food vacuoles, which contained a few loosely packed pigment crystals. In addition, the infected erythrocytes exhibited numerous knobs (essential for adhesion) at the erythrocyte surface (Figure 2a). In contrast, the HDL treated samples contained low numbers of infected erythrocytes and these contained predominantly early stages (approximately 60%) (Figure 2b) or dead parasites (Figure 2b, insert). Very few late stages

(approximately 3%) were observed (Figure 2c). The parasites were small, containing a single nucleus and no large food granules were observed, although a number contained pigment crystals in tight fitting vacuoles. The majority of infected erythrocytes possessed none or few knobs (Figure 2b). The dead parasites were characterised by having either electron lucent cytoplasm with disrupted membranes or were electron dense with loss of cytoplasmic detail, although clumps of pigment could be identified (Figure 2b, insert). The number of dead parasites appears to be related to HDL concentration and was less marked when lower concentrations were used.

Further evidence of the sensitivity of trophozoites to HDL was determined by incorporation of ^{3}H-hypoxanthine. Inhibition of incorporation of ^{3}H-hypoxanthine was achieved with HDL treated cultures at 24 hours after addition of HDL, i.e. within a single growth cycle; again illustrating that the toxic effect of HDL was on trophozoite maturation (Figure 1d).

Example 2

Hpr is the toxic factor responsible for HDL-mediated killing of *P. falciparum.*

Hpr has been demonstrated to be the active component of HDL that mediates killing of trypanosomes [1]. Based on this observation the inventors tested the hypothesis that the same protein may be responsible for the HDL-mediated killing of *P. falciparum*. HDL particles containing Hpr (but not haptoglobin) were passed over a column with bound polyclonal anti-haptoglobin (these antibodies cross-react with Hpr) ro remove Hpr. The resulting fraction was then dialysed and adjusted to the same total protein concentration as that in the original HDL fraction. The SDS-polyacrylamide gel shown in Figure 3a

demonstrates that Hpr was selectively removed from total serum HDL fraction. Sequencing of the 36 kDa protein (Figure 3a, lane 3) verified the presence of Hpr in the bound fraction. The toxic effects of HDL with and without Hpr were then compared. The removal of HDL containing Hpr totally abrogated the toxic effect of human HDL on parasite replication (Figure 3b). Light microscopic examination of Giemsa stained smears of cultures treated with HDL or HDL without Hpr or PBS showed that normal trophozoite and schizont maturation occurred in treatments with Hpr depleted HDL and in the PBS control whereas unhealthy trophozoites were observed in cultures treated with total serum HDL (data not shown). These experiments indicate that the subfraction of human serum HDL containing Hpr is the sole inhibitor of *P. falciparum* growth in human serum.

Haptoglobin has been shown to be an inhibitor of Hpr mediated killing of trypanosomes [8]. However, when haptoglobin was added to invasion assays at a range of concentrations 0 to 1.0 mg/ml, no inhibition of killing of *P. falciparum* was observed. These results are again consistent with the proposed differences in TLF binding to trypanosomes and *P. falciparum* infected erythrocytes.

Discussion

The Experimental examples included herein provide strong evidence that the human HDLs containing the apolipoprotein Hpr have a direct toxic effect on the development of the erythrocytic stages of *P. falciparum*. Hpr protein may be taken into *P. falciparum*-infected erythrocyte during maximal uptake of HDL by maturing trophozoites. Light microscopy and electron microscopy studies demonstrate that the toxic effect of Hpr is manifest during trophozoite

maturation (24 to 36 hours after erythrocyte invasion). HDL treatment appears to arrest the parasites and prevent development beyond the early trophozoite stage. The exact mechanism for this is unclear. There may be an effect on nutrient uptake because there was some evidence of abnormal membranes enclosing ingested haemaglobin. Defective food vacuole formation was also observed. These may be direct or indirect consequences of the toxic effect of Hpr on parasite development.

In *T. brucei brucei*, following endocytosis, the site of action of Hpr is the lysosome where toxicity is activated in the acidic conditions (<pH 4), resulting in oxidative damage to the membranes of lysosomes, leakage of their contents and autodigestion 7, 10. The food vacuole in *P. falciparum* is an acidic lysosome containing hydrolytic enzymes. It may be specialised only for haemoglobin degradation and processing of related breakdown products. By analogy with *T. brucei brucei*, it may be postulated that the food vacuole is the site of action of Hpr in *P. falciparum*.

P. falciparum digests haemaglobin in the food vacuole during trophozoite maturation as a source of amino acids and possibly iron, from 16 to 36 hours after invasion of the erythrocyte. The parasite has evolved specialised mechanisms to detoxify haem during digestion of haemoglobin by polymerisation of haem to form haemazoin pigment thereby preventing oxidative damage. The detoxification process involves the binding of parasite histidine rich protein II to haem. This catalyses the polymerisation of haem after haemoglobin digestion by specialised parasite proteases [12]. Light microscopy and EM studies lead us to speculate on two possible mechanisms of killing of the maturing trophozoite by Hpr. Firstly, transport

of this molecule to the food vacuole via HDL uptake mechanisms may lead to complexing of Hpr with released haem that could directly cause oxidative damage. Alternatively, Hpr may bind haemoglobin interfering with haemaglobin digestion and haem metabolism in the food vacuole. Inhibition of haem detoxfication would lead to parasite death by oxidative damage.

Inhibition of parasite growth in culture was efficiently achieved with HDL containing concentrations of trypanolytic activity in the middle to upper end of the range of physiological concentrations found in human serum (ie. 3000-17,000 TLF units per ml of serum). Variability of individual human sera to support the replication of *P. falciparum* in culture can be explained by proposing variable levels of Hpr. The concentrations of TLF required to kill *T. brucei brucei* (1-10 units/ml) are at least 3 orders of magnitude less than those required to kill *P. falciparum* (10^3-10^4 units/ml). Hpr sensitivity defines the complete refractoriness of humans to *T. brucei brucei*. The extreme sensitivity of *T. brucei brucei* to Hpr compared to *P. falciparum* can be best explained by the fact that *T. brucei brucei* is extracellular and TLF is concentrated directly to the lysosome. In contrast, susceptible stages of *P. falciparum* are intracellular and HDL containing Hpr must be trafficked through four membrane systems to reach the proposed site of action in the food vacuole.

The mechanism of uptake of Hpr by *P. falciparum* may be very different from that observed for *T. brucei brucei*. HDL is also required for *T. brucei brucei* growth. Cultivation of *T. brucei brucei* in Baltz-modified minimum essential medium with 10% lipoprotein-depleted fetal bovine serum required addition of purified HDL to the medium [13]. All serum HDL, both toxic and non-toxic, bind to *T. brucei*

brucei by a low-affinity high capacity apolipoprotein AI receptor within the trypanosome flagellar pocket (review [10], Drain and Hajduk, unpublished). However, binding to this receptor does not lead to internalization of HDL. This is analogous to the class B scavenger receptor SRB-1 described in mammalian cells and facilitates lipid exchanges between serum lipoprotein particles and cell membranes [14]. The cytotoxic effects of Hpr-containing HDL require binding to an Hpr receptor and subsequent internalization and lysosomal targeting (Drain & Hajduk, unpublished). HDL uptake mechanisms are not well defined for *P. falciparum* but it has been proposed that phospholipid exchanges occur by exchange mechanisms rather than endocytosis [11]. The observed toxic action of HDL against *P. falciparum* suggests that Hpr may be a defence against other Plasmodium species infecting humans as well as parasitic protozoa of diverse phylogenetic origins.

Host genetic data support a role for Hpr in protection against severe malarial disease. African-Americans have been found to have multiple copies of the Hpr gene [8].

Epidemiological observations of semi-immune children infected with multiple *Plasmodium* species show that total malaria parasitemia is regulated around a threshold value [15]. This density-dependant regulation of malaria parasitemia occurs by an induction and effector mechanism that is trans-species in action. The mechanism is not fever, but is likely to be mediated by the innate immune system [15]. Little is known about the mechanisms of production of Hpr. If produced at higher levels in response to parasite load by haemolysis or parasite toxin induced cytokines, it may play a key role in this type of non-specific parasite regulation. Vaccination strategies may be feasible to improve natural

mechanisms of immunity, if Hpr is under cytokine induction.

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Claims:

1. Use of an active substance comprising a mammalian haptoglobin-related protein or a functional equivalent, functional fragment or structural analog thereof for the manufacture of a medicament for use as an antiparasitic agent against intraerythrocytic parasitic protozoa or for use in the treatment or prevention of a condition associated with the presence of an intraerythrocytic parasitic protozoan in a host organism.

2. Use of an active substance comprising at least one of:
(i) a mammalian HDL containing haptoglobin-related protein,
(ii) a mammalian TLF1, or
(iii) a mammalian TLF2,
or a combination thereof, optionally in combination with a mammalian haptoglobin-related protein or a functional equivalent, functional fragment or structural analog thereof, for the manufacture of a medicament for use as an antiparasitic agent against intraerythrocytic parasitic protozoa or for use in the treatment or prevention of a condition associated with the presence of an intraerythrocytic parasitic protozoan in a host organism.

3. Use of a substance comprising a mammalian haptoglobin-related protein or a functional equivalent, functional fragment or structural analog thereof as an active pharmaceutical agent in the treatment or prevention of a condition associated with the presence of an intraerythrocytic parasitic protozoan in a host organism.

4. Use of a substance comprising at least one of:
(i) a mammalian HDL containing haptoglobin-related protein,
(ii) a mammalian TLF1, or
(iii) a mammalian TLF2,
or a combination thereof, optionally in combination with a mammalian haptoglobin-related protein or a functional equivalent, functional fragment or structural analog thereof as an active pharmaceutical agent in the treatment or prevention of a condition associated with the presence of an intraerythrocytic parasitic protozoan in a host organism.

5. Use as claimed in any one of claims 1 to 4 wherein the intraerythrocytic parasitic protozoan belongs to the genus *Plasmodium* or the genus *Babesia*.

6. Use as claimed in claim 5 wherein the host organism is a non-human organism.

7. Use as claimed in claim 5 wherein the intraerythrocytic parasitic protozoan is a *Plasmodium* spp., preferably *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* or *Plasmodium ovale*.

8. Use as claimed in claim 7 wherein the condition is malaria.

9. Use as claimed in any one of claims 1 to 8 wherein the mammalian haptoglobin-related protein, the HDL, the TLF1 or the TLF2 is of human origin.

10. Use as claimed in any one of claims 1 to 8 wherein the mammalian haptoglobin-related protein is of non-human primate origin.

11. A method of treating or preventing a condition associated with the presence of an intraerythrocytic parasitic protozoan in a host organism, which method comprises administering to a host organism in need thereof an effective amount of a medicament comprising an active substance comprising a mammalian haptoglobin-related protein or a functional equivalent, functional fragment or structural analog thereof and a pharmaceutically acceptable carrier, diluent or excipient therefor.

12. A method of treating or preventing a condition associated with the presence of an intraerythrocytic parasitic protozoan in a host organism, which method comprises administering to a host organism in need thereof an effective amount of a medicament comprising an active substance comprising at least one of:
(i) a mammalian HDL containing haptoglobin-related protein,
(ii) a mammalian TLF1, or
(iii) a mammalian TLF2,
or a combination thereof, optionally in combination with a mammalian haptoglobin-related protein or a functional equivalent, functional fragment or structural analog thereof, and a pharmaceutically acceptable carrier, diluent or excipient therefor.

13. A method as claimed in claim 11 or claim 12 wherein the intraerythrocytic parasitic protozoan belongs to the genus *Plasmodium* or the genus *Babesia*.

14. A method as claimed in claim 13 wherein the host organism is a non-human organism.

15. A method as claimed in claim 13 wherein the intraerythrocytic parasitic protozoan is a *Plasmodium*

spp., preferably *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* or *Plasmodium ovale*.

16. A method as claimed in claim 15 wherein the condition is malaria.

17. A method as claimed in any one of claims 11 to 16 wherein the mammalian haptoglobin-related protein, the HDL, the TLF1 or the TLF2 is of human origin.

18. A method as claimed in any one of claims 11 to 16 wherein the mammalian haptoglobin-related protein is of non-human primate origin.

19. A method of treating or preventing a condition associated with the presence of an intraerythrocytic parasitic protozoan in a host organism, which method comprises administering to a host organism in need thereof an effective amount of a medicament comprising an expression vector suitable for directing expression of a mammalian Hpr protein or a functional fragment or functional equivalent thereof in the host organism.

20. Use of a nucleic acid molecule encoding a mammalian haptoglobin-related protein or a functional fragment or functional equivalent thereof for the manufacture of a medicament for use as an antiparasitic agent against intraerythrocytic parasitic protozoa or for use in the treatment or prevention of a condition associated with the presence of an intraerythrocytic parasitic protozoan in a host organism.

21. A method of identifying a compound with potential antiparasitic activity against

intraerythrocytic parasitic protozoa, which method comprises the steps of:

(a) providing a recombinant host cell containing a reporter gene expression construct comprising the promoter region of the human HPR gene operably linked to a reporter gene;

(b) contacting said recombinant host cell with a test compound; and

(c) screening for expression of the reporter gene product.

wherein compounds which increase expression of the reporter gene product are scored as having potential antiparasitic activity.

22. A compound which is identifiable as having potential antiparasitic activity against intraerythrocytic parasitic protozoa using the method of claim 21.

23. The compound of claim 22 for use as an active pharmaceutical substance.

24. Use of the compound of claim 23 for the manufacture of a medicament for use as an antiparasitic agent against intraerythrocytic parasitic protozoa or for use in the treatment or prevention of a condition associated with the presence of an intraerythrocytic parasitic protozoan in a host organism.

25. Use of an antibody which binds preferentially to the human Hpr protein, as compared to the human haptoglobin protein, in a diagnostic test to determine the levels of Hpr protein in a sample taken from a mammalian subject.

26. Use of an antibody which binds preferentially to the human Hpr protein, as compared to the human haptoglobin protein, in a diagnostic test to determine whether an individual is likely to be susceptible or resistant to malaria.

27. An *in vitro* method of determining whether an individual is likely to be susceptible or resistant to malaria, which method comprises contacting a sample of tissue or body fluid from said individual with an antibody which binds preferentially to the human Hpr protein as compared to the human haptoglobin protein and detecting or quantitatively measuring any complexes formed by binding of said antibody to haptoglobin-related protein present in the sample of tissue or biological fluid.

28. A kit for use in carrying out the method of claim 27, the kit comprising an antibody which binds preferentially to the human Hpr protein, as compared to the human haptoglobin protein, and optionally means for detecting or quantitatively measuring complexes formed by binding of the said antibody to Hpr protein present in the biological sample.

Figure 1

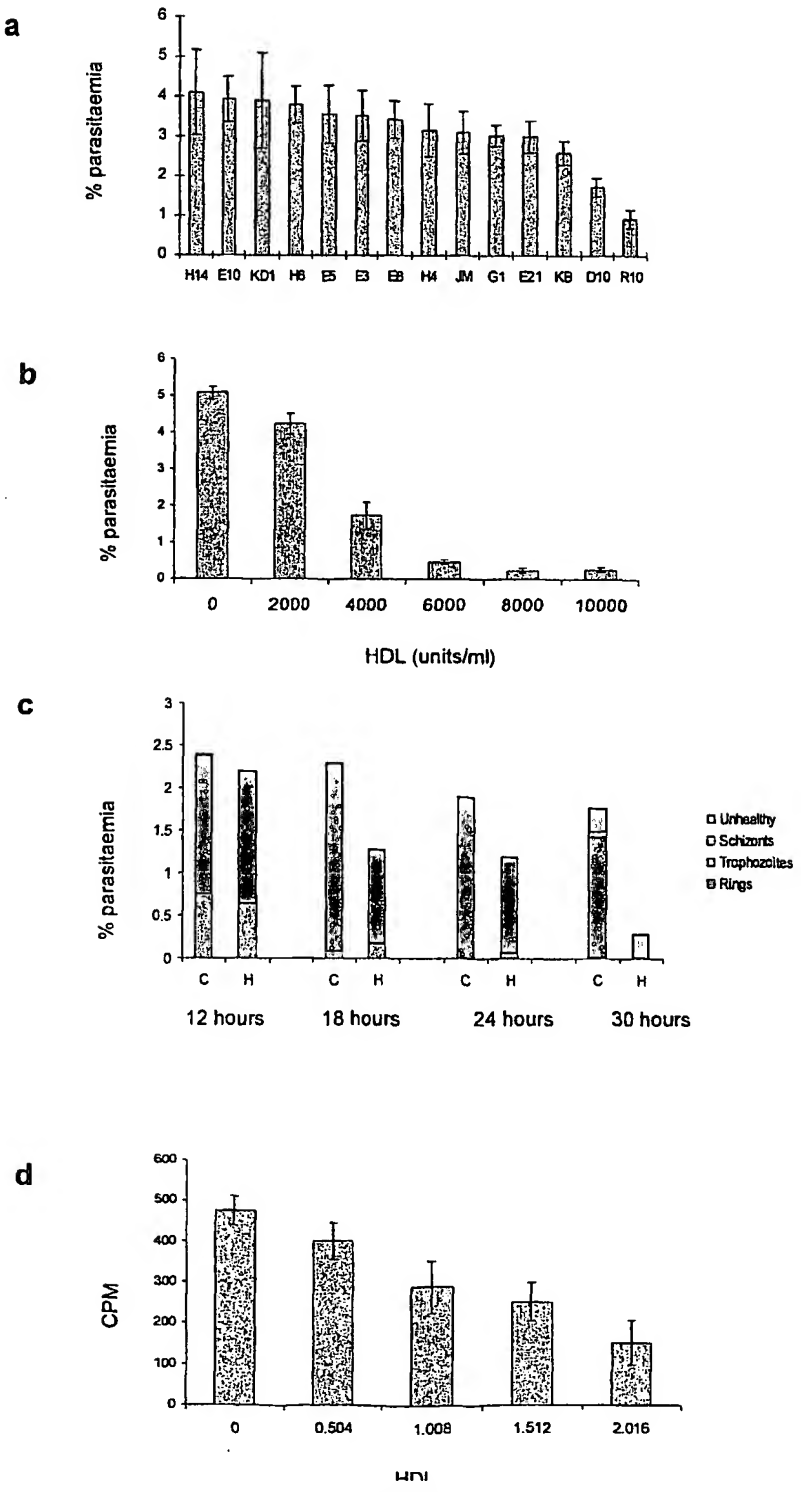
a
% parasitaemia
H14 E10 KD1 H6 E5 E3 E8 H4 JM G1 E21 K8 D10 R10
b
% parasitaemia
0 2000 4000 6000 8000 10000
HDL (units/ml)
c
% parasitaemia
Unhealthy
Schizonts
Trophozoites
Rings
C H C H C H C H
12 hours 18 hours 24 hours 30 hours
d
CPM
0 0.504 1.008 1.512 2.016

Figure 2

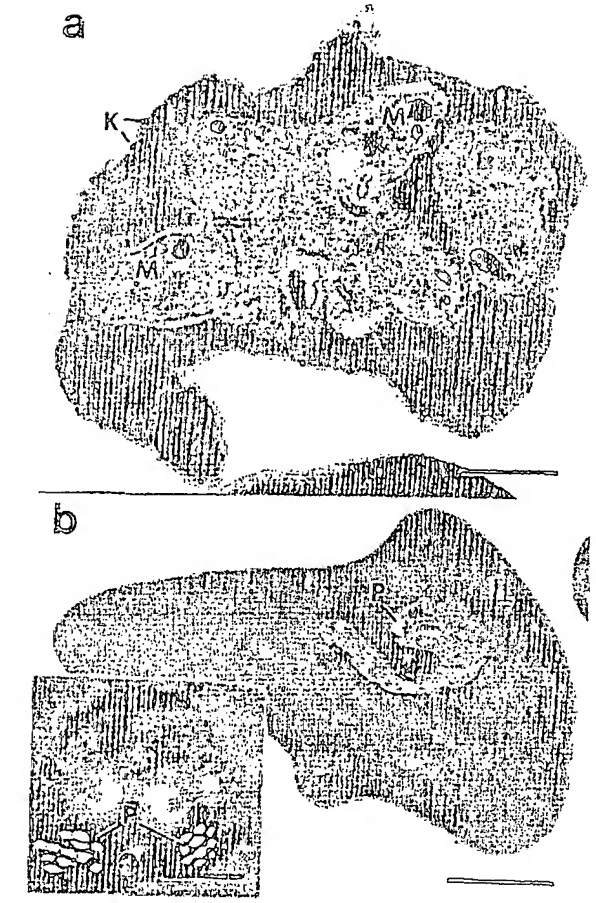
a
K
M
M
b
P
P
c
Relative no. of parasites
90
80
70
60
50
40
30
20
10
0
Control
HOL
Early Stages
Trophozoites
Dead parasites

Figure 3

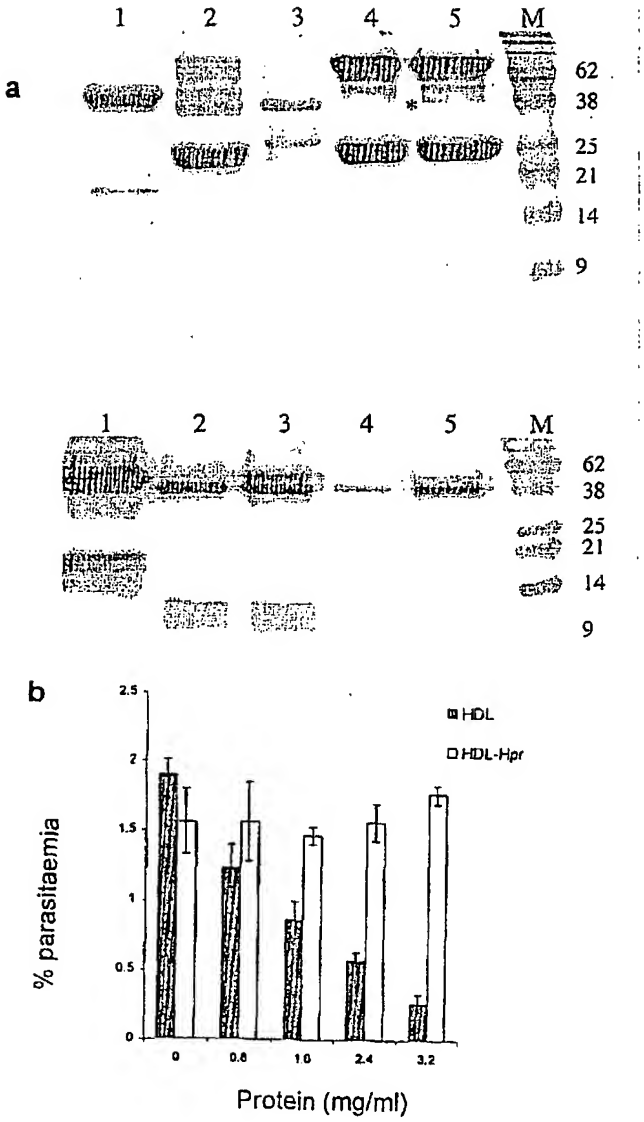
a
1
2
3
4
5
M
62
38
25
21
14
9
b
HDL
HDL-Hpr
% parasitaemia
2.5
2
1.5
1
0.5
0
0
0.8
1.6
2.4
3.2
Protein (mg/ml)

Figure 4

MSDLGAVISLLLWGRQLFALYSGNDVTDISDDRFPKPPEIANGYVEHLFRYQCKNYYRLRTEGDGVYTL
NDKKQWINKAVGDKLPECEAVCGKPKNPANPVQR*|ILGGHLDAKGSFPWQAKMVSHHNLTTGATLINEQ
WLLTTAKNLFLNHSENATAKDIAPTLTLYVGKKQLVEIEKVVLHPNYHQVDIGLIKLKQKVLVNERVMP
ICLPSKNYAEVGRVGYVSGWGQSDNFKLTDHLKYVMLPVADQYDCITHYEGSTCPKWKAPKSPVGVQPI
LNEHTFCVGMSKYQEDTCYGDAGSAFAVHDLEEDTWYAAGILSFDKSCAVAEYGVYVKVTSIQDWVQKT
IAEN